TECHNICAL REPORT

TR-76-33 FEL

RADIATION-INACTIVATION OF MEAT PROTEASES AS DETERMINED BY A ^{14}C-LABELED HEMOGLOBIN METHOD

Irradiated Food Products Group

Radiation Preservation of Food Division

November 1975

UNITED STATES ARMY
NATICK RESEARCH and DEVELOPMENT COMMAND
NATICK, MASSACHUSETTS 01760

Food Engineering Laboratory

FEL-48

Approved for public release; distribution unlimited.

Citation of trade names in this report does not constitute an official indorsement or approval of the use of such items.

Destroy this report when no longer needed. Do not return it to the originator.

DISCLAIMER NOTICE

THIS DOCUMENT IS BEST QUALITY AVAILABLE. THE COPY FURNISHED TO DTIC CONTAINED A SIGNIFICANT NUMBER OF PAGES WHICH DO NOT REPRODUCE LEGIBLY.

(19) TR-76-33-FEL (18) NARADCOM

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER: 76-33 FEL ✓	2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER	(9) Technical rept.
4. TITLE (and Subtitle): (6) RADIATION-INACTIVATION OF MEAT PROTEASES AS DETERMINED BY A ^{14}C-LABELED HEMOGLOBIN METHOD.	5. TYPE OF REPORT & PERIOD COVERED
	6. PERFORMING ORG. REPORT NUMBER: (14) FEL-48 ✓
7. AUTHOR(s): (10) G.W. Shults, J. S. Cohen [redacted] E./Wierbicki	8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS: US Army Natick Research & Development Command ✓ Natick, MA. 01760	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: (16) 6.2 1Y762724AH99
11. CONTROLLING OFFICE NAME AND ADDRESS: US Army Natick R & D Command, Natick, MA 01760 DRXNM-WR	(11) 12. REPORT DATE: Nov [redacted]75 (12) 20 p.
	13. NUMBER OF PAGES: 15
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report): Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Irradiation	Beef	Labeled Substrates
Irradiation Temperature	Pork	Labeled substances
Temperature	Chicken	Substrates
Meat	Proteases	
Enzymes	Radioactivity	

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

It was desired to study the effect of irradiation temperatures and dose on meat enzymes in fresh beef, pork, and chicken muscles. This was done using a rapid method for analysis which utilized a bovine hemoglobin substrate labeled with radioactive $K^{14}CNO$. The enzyme preparation was incubated with the ^{14}C-labeled substrate for 24 hours at 37°C. The amount of radioactivity recovered in the trichloracetic acid-soluble fraction after precipitation of the protein was used to measure the quantity of residual proteolytic enzymes (proteases). The sensitivity of the analyses was greatly improved by the substitution of

carbon-14 labelling

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

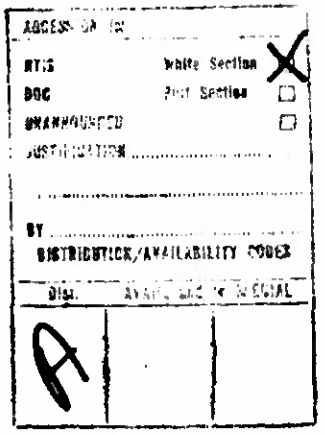
UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

408 903

1,4-dioxane in place of toluene as the diluent in the liquid scintillation of the precipitsted proteins.

Samplea of raw beef, pork, and chicken muscles were gamma-irradiated at doses of 2, 4, 6 and 8 Megarads and at irradiation temperatures of +21, 0, -30° and -80°C. The results showed a significant effect of both irradiation dose and temperature on the proteases. Irradiation with 2 Megarads at -80°C resulted in no reduction of proteolytic enzymes in beef, a 13% reduction in chicken, and a 13% reduction in pork. Irradiation with 8 megarads at +21°C results in a 86-91% reduction of proteolytic enzymes in the three muscles.



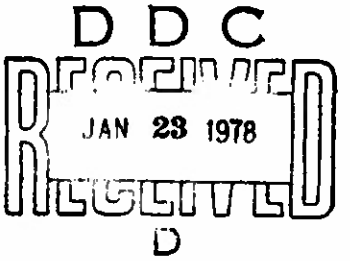


TABLE OF CONTENTS

PREFACE

These investigations were undertaken to determine the effects of irradiation dose and temperature on meat enzyme (proteases) of beef, pork and chicken muscle. The enzyme activity was determined using a radioactive ^{14}C-labeled bovine hemoglobin method. Results are reported as counts per minute detected in the filtrate of an acid precipitated reaction using a liquid scintillation counter.

The results show a significant effect of both irradiation dose and temperature on the three meats.

This study was undertaken as a research project of the Irradiated Food Products Group, Radiation Preservation of Foods Division, Food Engineering Laboratory, under Project 1Y762724AH99.

Radiation-Inactivation of Proteases as Determined by a ^{14}C-labeled Hemoglobin Method

INTRODUCTION

Radiation effects on the activity of the meat enzymes have been investigated since the initiation of the food irradiation program. The objective of early irradiation work was the production of a shelf-stable fresh meat item. However, it was found that the storage life of the irradiated fresh meats was limited due to breakdown of the nitrogenous constituents by the proteolytic enzymes (Doty and Wachter, 1955).[1] Thia enzymatic activity resulted in the development of off-flavors and a degradation of the texture of beef muscle.

Doty and Wachter (1955) found that irradiation at sterilizing doaes did not inactivate all the proteolytic enzymes. At a dose of 5×10^5 rep (1 rep equals 0.93 rad), a small reduction in the enzyme activity of beef was found; at an irradiation doae of 1.6×10^6 rep, a 50-percent reduction in the proteinase activity in beef muscle was found. Drake, et al. (1961)[2] found a decrease in preference ratings of raw ground irradiated beef that correlated to some extent with the action of endocellular tissue proteolytic enzymes.

All of the early worka on enzymes in irradiated meats used a colorimetric or spectrophotometric type assay to determine proteolytic activity. The data were expressed as tyrosine equivalent or as a percent change in nonprotein nitrogen. These procedurea were unsatisfactory due to poor reproducibility and the time required to obtain results.

1 - Doty, D. M., and J. P. Wachter 1959. Influence of gamma radiation on proteolytic enzyme activity of beef muscle. Jour. Agric. and Fd. Chem 3:61.

2 - Drake, M. P., G. D. Germon, and F. J. Kraus 1961. Proteolytic enzyme activity during storage of radiation-stabilized raw beef and its significance on flavor. Fd. Sci. 26:156.

Roth and Losty (1970)[3] and Roth et al.(1971)[4] developed a rapid method for the analysis of the meat enzymes. This method utilized hemoglobin labeled with $K^{14}CNO$ as the substrate. The amount of radioactivity recovered in the acid-soluble fraction after precipitation of the protein was used to measure the activity of the residual enzymes. The sensitivity of this teat is much greater than that of the colorimetric or spectrophotometric assaya because radioactivity can be detected at much lower levels with appropriate inatrumentation. Losty et al, (1973)[5] using the C^{14} labeled hemoglobin method found that increasing doses from 2 to 6 megarads destroyed up to 75% of the proteolytic activity in beef.

The objectives of these investigations were to improve the sensitivity of the analysis and determine the effects of irradiation dose and temperature on the proteolytic enzymes in beef, pork, and chicken muscles.

MATERIALS AND METHODS

Beef

The beef used for this study was fresh, 7 days postmortem, U.S. Commercial grade top round, (semimembranosus muscle-fat level 5-7%).

The meat was ground through a 5-mm grinding plate and uniformly mixed in a cold room (+2°C). The liver uaed was frozen calf liver.

3 - Roth, J. S. and T. Losty. University of Connecticut studies on improving a rapid method for determining proteolytic enzyme activity in irradiated meat. Contract No. DAA617-67-C-0158, Final Report. U. S. Army Natick Laboratories, Natick, MA 01760, 1970.

4 - Roth, J. S., T. Losty, and E. Wierbicki 1971. Assay of proteolytic enzyme activity using C^{14}-labeled hemoglobin. Anal. Biochem. 42:214.

5 - Losty, T., J. S. Roth, and G. W. Shults 1973. Effect of gamma irradiation and heating on proteolytic activity of meat samples. Jour. Agric. and Fd. Chem. 21:275.

Chicken

Fresh, 2-3-day old, boneless chicken breasts, skin and external fat removed, were used as the raw msterial. The breasts were ground with s 5-mm grinding plate, and after grinding, were uniformly mixed.

Pork

Fresh, 7 days postmortem. fresh hams (semimembranosus muscle) were used. The external fat was removed, and the hams (approx. 10% fat level) were ground through a 5-mm grinding plate in a mechanical grinder. After grinding, the meat was uniformly mixed in a cold room (+2°C).

Substrate Preparation

Four hundred mg of Bovine hemoglobin (Sigma Chemical Co., type 1 semi-purified) were dissolved in 15 ml of distilled wster and adjusted to pH 6.1 with 0.1N NaOH.

The volume was increased to 20 ml with distilled water and 0.4 ml (0.04m curie) of $K^{14}CNO$ was added. The hemoglobin was incubated at 50°C for 2 hours and allowed to stand 18 hours at +2°C. After 18 hours, 2 ml (20 μ-moles) of cysteine hydrochloride, pH adjusted to 6.1, was added and the substrate incubated at 37°C for two hours. The substrate was dialyzed in distilled water for 48 hours at +2°C.

Enzyme Preparation

Results as reported in Table 1 were obtained on two beef ssmples, liver and round, buffered at pH 3.8 and 5.5, and using two methods of tissue preparation. One-half of each test sample was homogenized using a Pyrex tissue culture-grinder with the grinding tube immersed in ice water. The remaining portion of each of the samples was homogenized using a Waring blender at high speed, with the grinding chamber cooled with ice wster.

Standard Tissue Assay

The standard enzyme assay using the labeled hemoglobin substrate is described by Roth et al. (1971)[4]. The enzyme solution (0.7 ml) was added to test tubes containing 1.4 ml of 0.2 M acetate buffer, pH 3.7, and 1 ml of substrate containing 16 mg of hemoglobin. The sample was incubated at 38°C in a shaker water bath. Aliquots (0.85 ml) were removed after 0 and 24 hours in most cases.

The aliquots of the enzyme and substrate mixture were treated with 0.2 ml of ice-cold 50% trichloroacetic acid to precipitate the protein, agitated in a Vortex mixer, and centrifuged in a refrigerated centrifuge at 2000 rpm for 30 min. The supernatant fraction was filtered through glass wool to remove any particles of substrate.

Radioactivity Assay

A 0.1 ml portion of the substrate enzyme mixture was added to 15 ml of a liquid scintillation mixture in 40-ml scintillation jars. The samples were counted in a Packard liquid scintillation counter for 10 minutes. Each sample was counted in 4 replicates at 0 and 24 hours incubation. Substrate blanks were run for each set of experiments; results on two types of scintillation solutions were obtained. Both of the scintillation solutions contained 1, 4,-bis-2, 5-phenyloxazolyl-benzene, 2,5-diphenyloxazole, naphthalene and 2-ethoxyethanol. The two solutions varied only in that one contained toluene and the other 1,4-dioxane.

Packaging and radiation Processing.

The ground meat aamples were packaged in 404x202 size cans at 16.6 kPa of pressure. Irradiation processing was performed in a cobalt-60 gamma source at the Radiation Laboratory, US Army Natick Research and Development

Command. Doses stated are the minimum dose and the ranges are the minimum dose plus 25%. Irradiation temperatures were controlled within $\pm 10^{\circ}C$ using a liquid nitrogen cooling system. Dose rate of the cobalt-60 source was 3.24×10^{4} J/(kg.sec).

RESULTS AND DISCUSSION

Effects of Test Methods

The procedures for assaying the meat enzymes were tested. Beef liver and muscle were used as the tissue samples with liver being very high in proteolytic enzymes and muscle being lower by 30-40%. Blending techniques, pH of the buffer solution, and composition of the scintillation counting solutions were investigated.

Two types of blending of the liver and muscle tissue samples were used, one with a pyrex tissue grinder, the other with a blender. The temperature of the tissue samples was controlled with ice water to minimize temperature effects of the grinding process. Tissue samples (420 to 440 mg/ml) were used for all the analyses. Table 1 shows the results of the testing of the method variables. In every case, the liver tissue sample had significantly higher counts than the muscle sample. Microgrinding with a tissue grinder was found to give higher counts per minute (CPM) on analysis with both scintillation systems. Microgrinding was more efficient in homogenizing the tissue sample and resulted in a better preparation for the enzymes to act upon the labeled substrate.

The pH level of the buffer for the enzyme-substrate mixture had a significant effect on the results of the analysis. Tissue-substrate mixtures buffered at pH 3.8 had significantly higher CPM's than mixtures

Table 1

Effect of test method variables on the radiological protease aaaay of beef liver and beef muacle.

Samples	pH, Buffer Solution	Scintillation Solution[1] Toluene	Dioxane
Beef Liver Microground[2]	3.8	781	10,356
Beef Liver Blender[3]	3.8	722	9,604
Beef Liver Microground	5.5	246	1,933
Beef Liver Blender	5.5	214	1,789
Beef Round Microground	3.8	422	4,217
Beef Round Blender	3.8	352	3,797
Beef Round Microground	5.5	143	565
Beef Round Blender	5.5	137	669
Substrate Blank		106	312

1 ~ Counts per minute, C^{14}, average of 4 replicates - 24 hr incubation

2 ~ Microground-homogenized with a tiaaue culture grinder.

3 ~ Blender-homogenized with a Waring Blender - high speed.

buffered at pH 5.5, the normal pH of the tissue samples. This held true for both scintillation counting solutions. This confirms previous data reported by Roth et al. (1971)[4].

The standard scintillation counting solution used previously for the enzyme assay contained toluene as the diluent, and the counts per minute were not high enough to show small differences between tissue samples. In an attempt to increase the sensitivity of the assay, 1,4-dioxane, which is used in other specific radiological assays, was substituted for the toluene. The use of 1,4-dioxane has been extensively investigated in biochemistry research because of its chemical solvent power when used in aqueous systems. Data in Table 1 show the significant improvements in the sensitivity of the assay. In microground beef liver, the CPM's were increased from 781, using toluene, to 10,356 with 1,4-dioxane. In beef muscle, which is much lower in total proteolytic activity, the CPM's were also significantly increased.

It was determined from the results listed in Table 1 that the analysis for residual meat enzymes should be done using microgrinding, buffered at pH 3.8, and dioxane in place of toluene in the scintillation counting solution.

Effects of Irradiation Temperature and Dose on Beef Enzymes.

The reduction in the enzyme activity of beef muscle irradiated at four doses and four irradiation temperatures are shown on Table 2. These data demonstrate a statistically significant effect of the irradiation dose and temperatures. At an irradiation dose of 2 Mrad -80°C, a 1.0% increase was obtained indicating that irradiation of the beef muscle at

Table 2 Effects of irradiation dose and temperature on the proteolytic enzymes of beef muscle.

Dose	Irradiation Temperature							
	+21		0		-30		-80	
kJ/kg	CPM[a]	%reduction[b]	CPM	%reduction	CPM	% reduction	CPM	% reduction
20	1733	57	2256	44	2712	33	4101	**
40	1416	65	1435	65	2216	45	2451	40
60	847	79	1140	72	2486	40	3342	18
80	538	86	727	82	1106	73	1621	60
Frozen control	4048							

[a] Counts per minute of 4 replications after 24 hours irradiation.

[b] Percent reduction from the frozen control sample.

** A 1% increase in counts per minute was obtained.

Analysis of variance

Dose F = 13.46 Significant (P <0.01)
Temperature F= 17.17 Significant (P <0.01)

this dose and temperature had little effect on the proteolytic enzymes; however, at 8 Mrad, -80°C, a 60% reduction in residual activity was found. At an irradiation temperature of +21°C and a dose of 2 Mrad, a 57% decrease in residual activity was found; at +21°C and 8 Mrad, the reduction of residual activity was 86%. The data on the effect of irrsdiation doses indicate that as the dose increased, the residual enzyme activity significantly decreased. Similarly, as the irradiation temperature was decreased from +21°C down to -80°C, the enzymes were protected from the damaging effects of the irrsdiation process and the resulting reduction in enzymatic activity was minimized.

Currently, the minimum 12-D sterilizing dose for irradiated beef is 3.7 Mrad at -30°C ± 10°C (Wierbicki and Heiligman, 1973)[6]. The data in Table 2 show that the proteolytic enzyme activity in rsw beef irradiated at this 12-D irradiation dose would be reduced about 45%. Consequently, these enzymes must be inactivated by other means, such as thermal treatments.

Effects of Dose and Temperature of Irradiation on Proteolytic Enzymes in Chicken

Irradiation dose and temperature effect on residusl enzyme activity of chicken muscle followed the same trends as found in the beef muscle. Table 3 lists the results of white chicken muscle irradiated at 4 doses and temperatures of irradiation. The resulting reduction in residual activity was found statistically significant for both dose and temperature. Stsndard

6 - Wierbicki, E. and F. Heiligman 1973. Shelf stable cured ham with low nitrite additions preserved by radappertization. Proc. Int. Symp. Nitrite Meat Prod., Zeist, Purdoc, Wageningen, The Netherlands.

Table 3 Effecta of irradiation dose and temperature on the proteolytic enzymes of white chicken muscle.

Dose	Irradiation Temperature							
	+21		0		-30		-80	
kJ/kg	CPM[a]	% reduction[d]	CPM	% reduction	CPM	% reduction	CPM	% reduction
20	2613	39	2249	48	3240	24	3737	13
40	1161	73	1227	71	2140	50	2770	35
60	589	76	1023	76	1248	70	1389	67
80	386	91	374	90	927	78	1219	69
Frozen control	1003							

[a] Counts per minute - average of 4 replications at 24 hours

[d] Percent incubation reduction from the frozen control sample

Analysis of Variance

Dose - F = 57.1 Significant (P <0.01)
Temperature F = 18.0 Significant (P< 0.01)

deviations (SD) for each of the samples are low, indicating the high reproducibility of the assay. The maximum reduction (90%) in the residual enzymatic activity was obtained using an irradiation dose of 8.0 Mrad at +21°C or 0°C. The minimum reduction (13%) in residual activity was obtained at 2 Mrad dosage and -80°C.

The minimum 12-D sterilizing dose for chicken muscle was determined to be 4.5 Mrad at -30°C by Wierbicki and Heiligman (1973)in reference 6. at 4-Mrad dose and temperature of -30°C, a 50% reduction of proteolytic enzyme activity was found. This corresponds with the data obtained on beef muscle. Again, the remaining proteolytic enzymes in the chicken muscle must be inactivated by a thermal treatment in order to store the product over an extended storage period at non-refrigerated temperatures without losing quality.

Effect of Irradiation Dose and Temperature on the Proteolytic Enzymes in Pork

Table 4 lists the results of pork muscle irradiated at 4 doses and 4 temperatures. The reductions in the enzyme activity of pork muscle as a result of irradiation dose and temperature followed the same trend as found in beef and chicken muscle. Both temperature and dose had a significant effect on proteolytic activity. The greatest reduction (89%) of enzyme activity was found using a dose of 8.0 Mrad at +21°C. The smallest reduction (30%) of enzyme activity was at a dose of 2 Mrad at -80°C. The effects of the irradiation temperature on enzyme activity at each dose are readily evident. Lowering the temperature of the meat during irradiation from +21°C down to -80°C resulted in a lowering of the percent reduction by as much as 40% in the case of products irradiated at 2 Mrad. This held true for all doses, but at the higher doses of 6 and 8 Mrad the decrease in the

Table 4 Effects of irradiation dose and temperature on the proteolytic enzymes of pork muscle.

Dose	Irradiation Temperature							
	+21		0		-30		-80	
kJ/kg	CPM[a]	%reduction[b]	CPM	% reduction	CPM	% reduction	CPM	% reduction
20	696	75	1078	62	1834	36	1989	30
40	584	79	690	76	1089	62	1751	39
60	459	84	542	81	890	69	953	67
80	253	89	340	88	544	81	1206	57
Frozen control	2851							

[a] Counts per minute - average of 4 replications at 24 hours incubation

[b] Percent reduction from the frozen control sample

Analysis of variance

Dose, F = 19.4 Sig. ($P < 0.01$)
Temperature F= 13.4 Sig. ($P < 0.01$)

percent reduction of enzyme activity was not as great.

The results obtained in the proteolytic enzyme assay of beef, pork, and chicken muscle clearly show the effects of the irradiation doses and temperatures on the proteases in the meat. Both irradiation dose and temperature were found to have significant effects on the residual protease activity. The maximum reduction in proteases of the three meats were noted at 8 Mrad, +21°C; beef 86%; pork and chicken, 88-90%. The least effects of irradiation dose and temperature were found at 2 Mrad -80°C. The remaining enzymes of the meat have to be inactivated by a thermal treatment to produce a stable irradiated product capable of storage at ambient temperatures over an extended period without refrigeration.

CONCLUSIONS

Samples of raw beef, pork and chicken muscles were gamma irradiated at doses of 2, 4, 6 and 8 megarads and at irradiation temperatures of +21°, 0°, -30°, and -80°C. The samples were analyzed for residue enzymes with a method which utilized a hemoglobin substrate labeled with radioactive $K^{14}CNO$.

The results showed a significant effect of both irradiation temperature and dose on the proteases. Irradiation at 2 megarads at -80°C resulted in no reduction in beef, 13% in chicken and 30% in pork. Irradiation with 8 megarads at +21°C resulted in an 86-91% reduction of the proteases in the three meats.

REFERENCES

1 - Doty, D. M., and J. P. Wachter 1959. Influence of gamma radiation on proteolytic enzyme activity of beef muscle. Jour. Agric. and Fd. Chem. 3:61.

2 - Drake, M. P., G. D. Germon, and F. J. Kraus 1961. Proteolytic enzyme sctivity during storage of radiation-stabilized raw beef and its significance on flavor. Fd. Sci. 26:156.

3 - Roth, J. S. and T. Losty. University of Connecticut studies on improving a rapid method for determining proteolytic enzyme activity in irradiated meat. Contract No. DAA617-67-C-0158, Final Report. U. S. Army Natick Laboratories, Natick, Massachusetts 01760, 1970.

4 - Roth, J. S., T. Losty, and E. Wierbicki 1971. Assay of proteolytic enzyme activity using C^{14}-labeled hemoglobin. Anal. Biochem. 42:214.

5 - Losty, T., J. W. Roth, and G. W. Shults 1973. Effect of gamma irradiation and heating on proteolytic activity of meat samples. Jour. Agric. and Fd. Chem. 21:275.

6 - Wierbicki, E. and F. Heiligman 1973. Shelf stable cured ham with low nitrite additions preserved by radappertization. Proc. Int. Symp. Nitrite Meat Prod., Zeist, Purdoc, Wageningen, The Netherlands.